A Method for Counting Microorganisms in Maple Sirup*

Maple sirup, which is composed primarily of sucrose, is subject to attack by a variety of microorganisms. The growth of bacteria may result in the "souring" of the sirup, or the production of polysaccharide "slimes." Yeast growth can produce gas in closed containers, often with enough pressure to cause an explosive rupture. Mats of mold mycelia growing on the surface are unsightly, and the metabolic activities of the molds affect the quality of the sirup. All three groups of microorganisms produce a deleterious flavor effect on the sirup.

Contamination of the sirup with bacteria, yeast, or molds generally occurs after it is made. In producing the sirup, maple sap is concentrated 35 to 40-fold at temperatures above boiling water. The combined effect of the heat and the high concentration of sugar is usually sufficient to sterilize the sirup. Improper storage and handling procedures, however, allow microorganisms access to the sirup, and further development of the organisms depends on the establishment of conditions favorable to their growth.

A quantitative microbiological evaluation of the viable forms of bacteria and mold in maple sirup has never been reported in the literature. However, the presence of yeast (1) and molds (2, 3) in sirup has been described, and the occurrence of "sour" sirup due to the action of bacteria is well known in the industry. Knowledge of the number of viable organisms in a sirup would be useful in the manufacture of a higher quality product.

Although methods for counting yeast, molds, and bacteria in maple sirup have not been described, it may be assumed that the standard bacteriological plating techniques could be used for counting the microorganisms. In recent years, however, membrane filters have proved to be more desirable in the determination of the microbial contents of liquids and gases. In the cane sugar and sirup industry, membrane filter procedures have been proposed. Coleman and Bender (4) have shown that there is an 0.96 correlation between the two methods. Yeast counts in sugar liquors were made by Moroz (5), and the greater reliability of the membrane procedure with samples containing few microorganisms was demonstrated.

The microorganisms prevalent in cane sugar sirups are not the same as those found in maple sirup, so it is necessary to establish methods for the cultivation of the latter organisms. This report describes the preliminary procedures developed in this laboratory to count the number of bacteria and molds present in maple sirup. The microorganisms are collected and grown on a membrane filter, and after short incubation times, the colonies are counted directly.

Experimental Procedure

Apparatus (see Fig. 1).—The filter holder consists of a stainless steel funnel and a wire mesh filter support. The membrane filter is a disc of porous cellulosic material 47 mm in diameter. Approximately 80% of the volume

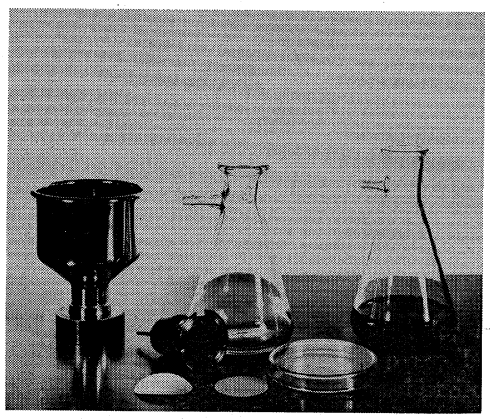

Fig. 1—Apparatus required for the determination of microorganisms by membrane filtration.

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of the filter is composed of uniform pores, 0.45 mμ. These filters may be plain white or may have a grid imprint to aid counting. Absorbent pads approximately the same size as the membrane filter are used as a supporting structure for nutrient media and staining solutions. Petri plates of either glass or plastic can be used. The plastic plates are less expensive than glass, and can be re-used for the membrane filter technique.

Sterilization of Equipment.—The filter holder was not sterilized but was washed for several minutes in very hot water between samples. Another procedure recommended for reducing contamination from the filter holder involves submerging the filter support and funnel in an antiseptic solution for several minutes, then washing in boiling water.

The membrane filters and absorbant pads were placed in separate glass petri plates and sterilized at 121°C (15 psi) for 15 minutes. The autoclave pressure was lowered rapidly to reduce condensation of moisture on the filters.

The glass petri plates were sterilized with dry heat (180°C) for 3 hours. (Plastic petri plates are obtainable in sterile condition in plastic bags. For re-use, the plastic plates can be sterilized by exposure to ultraviolet light for 30 minutes.)

Preparation of Sample.—For bacterial counts the liquid media consisted of tryptone (1%), glucose (2%), and Difco beef extract.[1] Mold counts were made in 6% Sabouraud's Liquid Medium (Difco). Both media were sterilized at 121°C for 15 minutes.

Maple sirup was too viscous to pass through the membrane filter readily. Dilutions were made to facilitate filtration and to reduce the number of organisms per ml so as to prevent overloading of the filter where high microbial counts were expected. (For greater reliability and ease in counting, the filters should contain less than 100 colonies.) Since there was no limit to the volume of sample that could be filtered, sirups with low microbial population could be counted accurately by increasing the volume of sample put through the filter. In the tests reported, 50 ml of maple sirup was added by a large-bore pipet to 450 ml of sterile distilled water. After the sirup dilution was thoroughly mixed, 50 ml aliquots were removed to sterile 50 ml graduates for filtration.

[1] Mention of trade names does not imply endorsement by the Department over others not named.

Procedure.—The wire mesh filter support was inserted in a one-hole rubber stopper which was placed in the mouth of a suction flask. A sterile membrane filter was placed on the mesh filter support and clamped in place with the locking ring at the base of the funnel. (The filters and absorbent pads were handled with forceps which were kept in 95% alcohol and flamed before use.) The sample was poured into the funnel and vacuum was applied until filtration was complete. The funnel was removed, and the filter was taken off the mesh support and placed on an absorbent pad in a sterile petri plate. (The absorbent pad was prepared before use by the addition of about 2 ml of the appropriate medium.)

Table 1. Replicate bacterial counts of a sample of maple sirup[a]

Sample	Days		
	1	2	3
1	12	7	9
2	11	11	7
3	6	14	7
4	5	12	13
5	5	8	18
6	10	15	6
7	17	20	6
8	22	19	9
9	19	15	16
10	10	–	14
$\bar{x}$[b]	11.7	13.4	10.5
s	6.3	4.45	4.4
$S_{\bar{x}}$	1.99	1.15	1.22

$\bar{x} \pm t_{.05} S_{\bar{x}} = 11.7 \pm 2.07$

[a] Bacterial count per 5 ml of sirup.
[b] $\bar{x}$ = mean bacterial count; s = standard deviation; $S_{\bar{x}}$ = standard error of the mean.

Cultivation and Evaluation.—The plates were incubated at 30°C. Bacterial colonies developed in 18–24 hours, and the mold colonies were countable in 36–48 hours.

For greater ease in counting bacterial colonies the following staining method is employed: An absorbent pad is saturated with about 2 ml of a 2% solution of triphenyl tetrazolium chloride. The filter is transferred from the nutrient medium pad to the staining pad. Within 5 minutes the colonies turn red as a result of the formozan produced by the

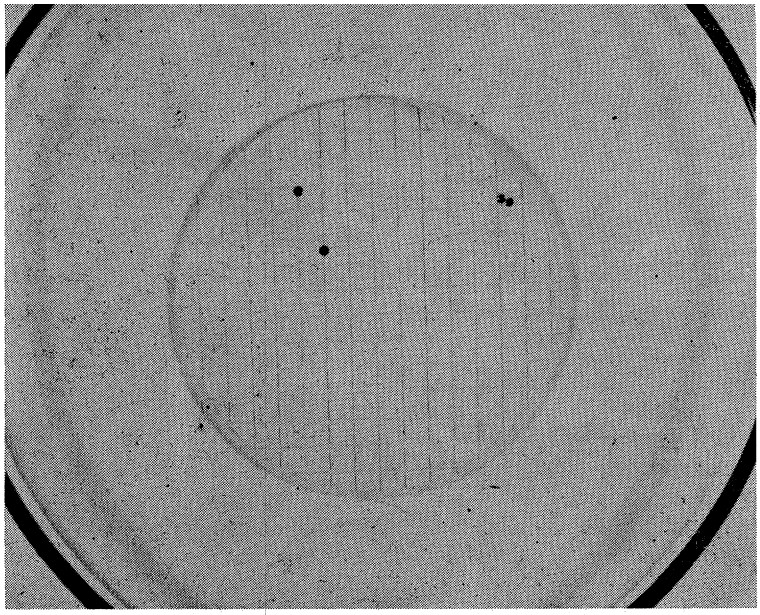

Fig. 2—Bacterial colonies growing directly on the membrane filter; after staining with triphenyl tetrazolium chloride.

reduction of the dye that diffuses through the filter, and a direct count can readily be made.

The appearance of the filter with bacterial colonies is shown in Fig. 2. Fig. 3 shows the number of mold spores originally present; Fig. 4 shows a filter containing both mold and bacteria.

Results and Discussion

Results of a series of replicate analyses of the bacterial count of a single lot of maple sirup are shown in Table 1. An analysis of variance of the data is shown in Table 2.

There is no significant difference at the 1% level in the values of the bacterial counts obtained on the different days. An analysis of the variance of the counts within a day could not be made because each determination was not an individual sample of the sirup.

Table 2. Analysis of variance of the replicate bacterial counts from a sample of maple sirup[a]

Source	D.f.	S.S.	M.S.	F
Total	28	590	21	—
Between days	2	41	20	0.95
Error	26	549	21	—

[a] No significance at the 1% level.

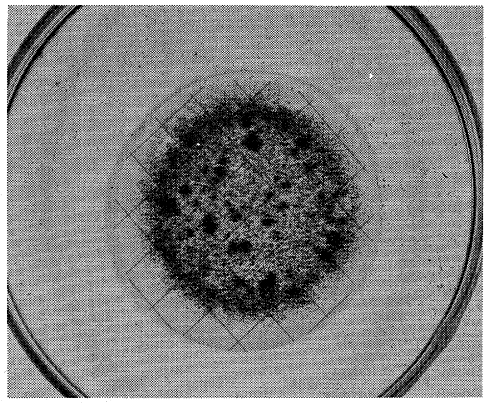

Fig. 3—Mold colonies growing directly on the membrane filter.

Although mold counts were not carried out so that statistical analyses could be made from the data, the experiments indicate that the membrane filter can be used for counting mold spores in maple sirup.

The technique of handling the membrane filtration apparatus is not complicated and can be learned rapidly by laboratory personnel with limited technical experience.

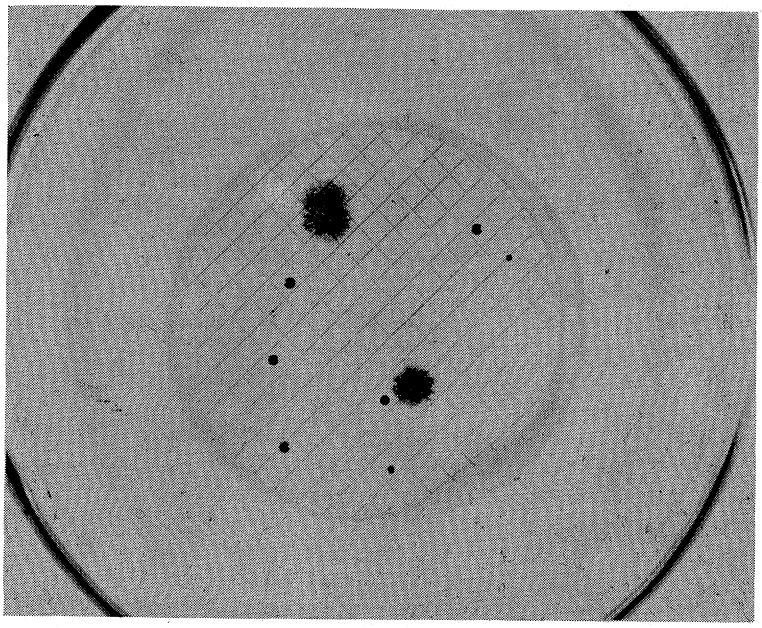

Fig. 4—Mold and bacterial colonies growing on the membrane filter; after staining with triphenyl tetrazolium chloride.

Summary and Recommendation

A method is described for obtaining the count of viable yeast, bacteria, and molds present in maple sirup. Appropriate dilutions of sirup are filtered through membrane filters, and the impinged microorganisms are grown *in situ* on the filter placed on an absorbent pad saturated with the proper media. Bacterial colonies can be counted in 18–24 hours after staining with triphenyl tetrazolium chloride; mold colonies can be counted in 36–48 hours. A statistical analysis of replicate bacterial counts indicates the reliability of the membrane filter method in determining the number of bacteria in maple sirup.

It is recommended[2] that collaborative studies be conducted on the counting methods for microorganisms in maple sirup.

REFERENCES

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(3) Heald, F. D., and Pool, V. W., Nebraska Agricultural Experiment Station, 21st Annual Report, 1908, p. 54.
(4) Coleman, M. C., and Bender, C. R., Society of Soft Drink Technologists, 4th Annual Meeting, 1957, pp. 126–137.
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[2] This recommendation was approved by the General Referee and by Subcommittee D, and was adopted by the Association. See *This Journal,* **45**, 129 (1962).